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Neurobehavioral changes and activation of neurodegenerative apoptosis on long-term consumption of aspartame in the rat brain

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ABSTRACT

Though several studies on toxic effect of aspartame metabolite have been studied, there are scanty data on whether aspartame exposure administration could release formate, a methanol metabolite thereby inducing oxidative stress and neurodegeneration in brain discrete region. To mimic the human methanol metabolism, the methotrexate (MTX) treated folate deficient rats were used. Aspartame was administered orally to the MTX treated animals and was studied along with controls and MTX treated controls. Oral intubations of FDA approved 40 mg/kg b.wt aspartame were given daily for 90 days. The loco–motor activity and emotionality behavior in the aspartame treated animals showed a marked increase in the immobilization, fecal bolus with a marked decrease in ambulation, rearing, grooming. The anxiety behavior in the aspartame treated animals showed a marked decrease in percentage of open arm entry, percentage of time spent in open arm and number of head dips. It is appropriate to point out, formaldehyde and formate could have led to an increased formation of free radical in the aspartame treated animals resulting in altered neurobehavioral changes owing to neuronal oxidative damage. Aspartame induced ROS may be also linked to increased neuronal apoptosis. In this study the aspartame treated animals showed an up regulation in the apoptotic gene expression along with protein expression in the respective brain region indicating the enhancement of neuronal cell death. This study intends to corroborate that chronic aspartame consumption can alter the behavior and neurodegeneration in brain discrete regions.

1. Introduction

A commonly used low calorie artificial sweetener Aspartame, (L-aspartyl-L-phenylalanine methyl ester) discovered in the year 1965 by James Schlatter of the G.D. Searle Company. The increased market of dietary products and the development of new synthetic sweetening compounds have not been sufficiently explored. Thus, to verify the risks and benefits of a substance present in our day-by-day use like aspartame, leads us to worry about the actions of its metabolites (aspartic acid, phenylalanine and methanol) to our organism [1]. Even after the commercial approval of aspartame by the FDA from the year 1981 [2] 40% of all complaints issued to the FDA have been concerning adverse reactions after consumption of aspartame [3] and since then it has caught the attention of many researchers. Upon ingestion aspartame molecule is metabolized into three metabolites namely 50% is phenylalanine, 40% is aspartic acid, and 10% is methanol. The first two are known as amino acid isolates. It has been reported that consumption of aspartame could cause neurological and behavioural disturbances in sensitive individuals [4]. Large doses of both aspartame as well as these individual metabolites have been tested in humans and other animals producing a controversial report. It has been reported that not only the metabolites of methanol but methanol per se as well is toxic to the brain [5]. The primary metabolic fate of methanol is the direct oxidation to formaldehyde and then into formate. The toxic effects of methanol in humans are due to the accumulation of its metabolite formate [6]. Relatively small amount of aspartame can significantly increase methanol levels [7], being that 10% of this metabolism results in methanol, which is oxidized to formaldehyde and formate in many tissues. Several studies on laboratory animals have been made to verify aspartame's toxicity. Recently, a very large experiment confirmed that it is a multipotential carcinogenic agent when given at a daily dose of 20 mg/kg body weight, an amount well below the acceptable daily dose of 40 mg/kg body weight [8].

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Formate is metabolized twice as fast in the rat as in the monkey [9]. The rodents do not develop metabolic acidosis during methanol poisoning, owing to their high liver folate content and in order to create similar results in human beings only folate deficient rodents are required to accumulate formate in order to develop acidosis [10]. Hence, in this study in order to mimic the human situation, a folate deficiency status is induced by administering MTX. Relatively small amount of aspartame can significantly increase methanol levels [11].Methanol is being increasingly recognized as a substance that damages the liver cells where it is oxidized to formaldehyde and latter to formate [12]. Based on the literature study Kruse [13] suggested that among the metabolites, methanol is a toxicant that causes systemic toxicity. Parthasarathy et al. [11], reported that methanol is primarily metabolized to formaldehyde and then to formate, accompanied by the formation of superoxide anion and hydrogen peroxide. Ashok et al. [14], have also reported that chronic methanol exposure, which is a byproduct of aspartame, may be responsible for the alteration observed in the free-radical-scavenging system. Of all the organs in the body, the CNS is rich in oxygen which exceeds its share of oxidative abuse due to free radicals. This is associated with the abundance of redox active transition metal ions and the virtual decease of antioxidant defense system [15], the effect of oxidative modification by reactive oxygen species (ROS) on neuronal phospholipids, DNA, and proteins has been implicated in the genesis of several neurodegenerative disorders [16]. Potts et al. [17], showed that the administration of aspartame as 9% of the diet for 13 weeks could alter learning behavior in rats. Ashok et al., reported that methanol released by aspartame has an effect in the brain with an observed change in the locomotor and anxiety level [18].

Recent studies on aspartame have been carried out to understand the mechanisms of neurotoxicity [19,20]. The apoptotic process is also affected by many other signaling pathways, especially activation via ROS in TNF- α-mediated JNK c-Jun N-terminal kinases (JNK) (stress activated protein kinases) which has been consistently demonstrated in various cellular systems [21]. JNK3 is expressed predominantly in neurons but also in cardiac smooth muscle and testes, which has 2 isoforms. Several different but coexisting mechanisms guarantee the specificity of JNK in signal transduction [22]. It is important to emphasize that in some instances of neuronal injury, sustained c-Jun induction/activation does not necessarily lead to death [23]. JNK activation in a primary role from where it may induce expression of Fas or TNF-α [24] to commit cells to apoptosis. JNK3 is predominately expressed in the brain and is most consistently associated with neuronal death.

The Fas/FasL system transmits apoptotic signals from the surrounding environment into the cell. Fas contain a single transmembrane domain and belong to the tumor necrosis factor (TNF)/nerve growth factor family [25]. FasL contains a single transmembrane domain and is also a member of the same TNF family [26]. A soluble form of FasL has been described, but appears to be less capable of inducing apoptosis, when compared with the bound form [27]. The binding of FasL with Fas initiates receptor oligomerization, which recruits Fas-associated death domain (FADD) [28]. FADD binds procaspase-8 and permits activation of caspase-8 through self-cleavage [29]. Caspase-8 activates the effector caspases, which commits the cell to the orderly process of apoptosis [30]. A transient and modest JNK activation mediates cell survival via NF-κB- induced apoptotic gene expression, whereas a prolonged and robust JNK activation is associated with cell apoptosis via ASK1 signaling [31]. This study is designed to determine whether the chronic oral administration of aspartame (40 mg/kg) can release methanol as a by-product after its metabolism and the effect of aspartame on receptor mediated Fas pathway on neuronal apoptosis in the brain regions and its role in anxiety and emotional behavior.

2. Materials and methods

2.1. Animals

Wistar strain male albino rats (200–220 g) were maintained under standard laboratory conditions with water and food. For the folate-deficient group, folate-deficient diet was provided for 45 days prior to the experiment and Methotrexate (MTX) was administered for a week before the oral intubation of aspartame. The folate deficiency was confirmed by monitoring the FIGLU level in the urine, after this confirmation the aspartame oral intubation was started. The animals were handled according to the principles of laboratory care framed by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Prior to the experimentation, proper approval was obtained from the Institutional Animal Ethical Committee (No: 01/032/2010/Aug-11).

2.2. Chemicals

Aspartame and Methotrexate were purchased from Sigma aldrich, St. Louis, MO, USA. Secondary antibody was purchased from Merck, Bangalore, the primary antibody was purchased from Biovision, Pierce USA. Taq-Polymerase, DNTPs from (Genet Bio) China, RT enzyme kit from (Thermo scientific, USA) and other molecular grade chemicals from Merck Bangalore, India. All other chemicals were of analar grade obtained from Sisco research Laboratory, Bombay, India.

2.3. Experimental design

2.3.1. Aspartame dose

The European Food Safety Authority recently confirmed its daily acceptable intake (ADI) for aspartame of 40 mg/kg b.wt./day. Aspartame mixed in sterile saline was administered orally (40 mg/kg body weight) for 90 days and this dosage was based on the FDA approved Daily acceptable intake (ADI) limit.

2.3.2. Groups

The rats were divided into three groups, namely, saline control, MTX-treated control, and MTX-treated aspartame administered groups. Each group consisted of six animals. One separate set of animals were employed for behavioral studies. One set of animals was used for biochemical assays. One set of animals were used to study the gene and protein expression, One set of animals were used to study the histology and immunohistochemistry.MTX in sterile saline was administered (0.2 mg/kg/day) subcutaneously for 7 days to induce folate deficiency in MTX controls as well as to MTX + aspartame treated groups [32]. One week after treatment with MTX, folate deficiency was confirmed by estimating the urinary excretion of formaminoglutamic acid (FIGLU) [33]. From the eighth day, the MTX-treated aspartame group received the oral intubation of aspartame, whereas the other two groups received equivalent volumes of saline as an oral dose and all animals were handled similarly. The chronic dose of aspartame was given for 90 days and all the animals were fed folate-deficient diet except the control animals till 90 days.

2.4. Sample collections

The animals were sacrificed using higher dose of long acting pentothal sodium (100 mg/kg.b.wt). The blood samples and isolation of brain was performed between 8 and 10 a.m. to avoid circadian rhythm induced changes. The brain was immediately removed and washed with ice-cold phosphate buffered saline